A novel protein phosphatase indirectly regulates phytochrome-interacting factor 3 via phytochrome

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Light signal transduction in plants involves an intricate series of pathways which is finely regulated by interactions between specific signalling proteins, as well as by protein modifications such as phosphorylation and ubiquitination. The identification of novel phytochrome-interacting proteins and the precise signalling mechanisms that they mediate is still ongoing. In our present study, we show that the newly identified putative phytochrome-associated protein, PAPP2C (phytochrome-associated protein phosphatase type 2C), interacts in the nucleus with phyA (phytochrome A) and phyB, both in vitro and in vivo. Moreover, the phosphatase activity of PAPP2C and its association with phytochromes were found to be enhanced by red light, indicating that it plays a role in mediating phytochrome signalling. In particular, PAPP2C specifically binds to the N-terminal PHY domain of the phytochromes. We thus speculate that this interaction reflects a unique regulatory function of this phosphatase toward established phytochrome-associated proteins. We also show that PAPP2C effectively dephosphorylates phytochromes in vitro. Interestingly, PAPP2C indirectly mediates the dephosphorylation of PIF3 (phytochrome-interacting factor 3) in vitro. Taken together, we suggest that PAPP2C functions as a regulator of PIF3 by dephosphorylating phytochromes in the nucleus.

Key words: Arabidopsis thaliana, dephosphorylation, phosphatase, phytochrome, phytochrome-associated protein phosphatase type 2C (PAPP2C), phytochrome-interacting factor 3 (PIF3)

INTRODUCTION

Plant light signal transduction is a highly complex process and is initiated by the three major classes of photoreceptors: the phytochromes, the cryptochromes and the phototropins [1–4]. Among these, the phytochromes are conjugates of the PHY apoprotein, which is covalently attached to a linear tetrapyrrole phytochromobilin chromophore [5]. In Arabidopsis, phytochromes are encoded by five genes, PHYA to PHYE, the protein products of which are expressed in the dark as the inactive red-light-absorbing Pr forms. Conversion of these inactive precursors into active far-red-light-absorbing Pfr forms subsequently occurs under specific wavelengths of light. The Pfr form of phyA (phytochrome A) is degraded rapidly upon light exposure, whereas phyB–phyE are light-stable in both forms [6].

The N-terminal domains of the phytochromes contain a chromophore-binding site and perceive light signals. The C-terminal domains contain dimerization motifs, two PAS (Per/Arnt/Sim) domains and a HKRD (histidine kinase-related domain), and are responsible for transducing light signals to downstream components [7]. Red (660 nm) or far-red (730 nm) light photo-reversibly isomerizes the phytochromobilin chromophore and causes subsequent changes to the protein structure (Pr/Pf) and subcellular localization [4]. In recent years, many components involved in the phytochrome signalling pathways have been screened in genetic studies [8] and yeast two-hybrid assays [9–12]. In our previous experiments, we have identified several phytochrome-interacting proteins from plant tissues by immunopurification-coupled MS [13].

Light signalling mechanisms in plants are usually modulated by various post-translational modifications such as phosphorylation/dephosphorylation [14]. Phytochromes can function as serine/threonine kinases due to the conserved HKRD in their C-terminal regions [15,16] and can phosphorylate a number of phytochrome-interacting proteins including PKS1 [11], Aux/IAA [17] and the cryptochromes 1 and 2 [18]. Recently, PIF (phytochrome-interacting factor) 3 and PIF5 have also been reported to be phosphorylated by a photo-activated phytochrome [19,20]. The phytochromes are also phosphorylated in vivo and in vitro [21,22], which is enhanced by light illumination [16]. The Ser1 and Ser598 residues of oat phyA have been shown to be phosphorylated in vivo, whereas Ser17 and Ser198 are phosphorylated by PKA (protein kinase A) in vitro. The Ser7 residue is phosphorylated in both the Pr and Pfr forms of phyA, whereas Ser598 is preferentially phosphorylated in the Pfr form [16,22,23].

A hypersensitivity to far-red light has been observed from plants expressing oat phyA harbouring a deletion of its N-terminal serine-rich region, including Ser1, suggesting that the phosphorylation of this N-terminal domain regulates the activity of this phytochrome [24–26]. For higher-plant phytochromes, the phosphorylation of Ser198 is also suggested to be important for physiological events such as the affinity for downstream signalling transducers [27,28]. It is postulated from these findings

Abbreviations used: BiFC, bimolecular fluorescence; CAB1, chlorophyll a/b binding protein 1; CHS, chalcone synthase; DAPI, 4′,6-diamidino-2-phenylindole; GAF, cGMP-specific phosphodiesterases cyanobacterial gdenylate cyclases, and formate hydrogen lyase transcription activator FhlA; GST, glutathione transferase; GUS, β-glucuronidase; HKRD, histidine kinase-related domain; KP, kinase/phosphatase; MBP, maltose-binding protein; NTE, N-terminal extension; PAPP2C, phytochrome-associated protein phosphatase type 2C; PAS, Per/Arnt/Sim; phyA etc., phytochrome A etc; PIF3, phytochrome-interacting factor 3; PKA, protein kinase A; PP, protein phosphatase; PRD, PAS-related domain; T-DNA, transfer DNA; YFP, yellow fluorescent protein.

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that the fine-tuning of the phosphorylation/dephosphorylation of phytochromes and the subsequent phosphorylase to their downstream components are required for their proper function [29]. However, the protein kinases responsible for the phytochrome phosphorylation have not been identified thus far, although two phytochrome-associated PPs (protein phosphatases) have been isolated [28,30]. Moreover, since these light signal transduction mechanisms have been studied using a recombinant oat phyA in vitro, the in vivo machinery in plants remains to be defined. In our present study, we demonstrate that a novel phytochrome-associated PP, PAPP2C, indirectly controls the phosphorylation of PIF3 via the dephosphorylation of phytochromes.

EXPERIMENTAL

Construction and purification of recombinant PAPP2C protein

For the cloning of the PAPP2C gene, total RNA was isolated from wild-type Arabidopsis (Col-0) seedlings grown under darkness for 4 days. Single-stranded cDNA was then synthesized using a first-strand cDNA synthesis kit for RT (reverse transcriptase)–PCR (Roche). The PAPP2C gene was subsequently amplified from the resulting cDNA pools using specific primers, subcloned into the pGEX-4T-2 Escherichia coli expression vector (GE Healthcare) and transformed into E. coli BL21 cells. The BL21 cells were then grown at 37°C to an OD600 of 0.6, and recombinant protein expression was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) at 37°C. After 3 h induction, the cells were harvested by centrifugation at 5000 g for 5 min at 4°C and resuspended in 0.02 culture vol. of cold lysis buffer [1% (v/v) Triton X-100, 1 mM EDTA and protease inhibitor cocktail (Roche)]. For fusion protein purification, crude extract was applied to glutathione–Sepharose 4B beads (GE Healthcare) followed by elution with a buffer containing 50 mM EDTA and 0.1% (v/v) 2-mercaptoethanol and 4 mM MgCl2 overnight at 4°C. The purified recombinant PAPP2C was stored at −80°C until required.

Generation of mutant and transgenic plants

A homozygous T-DNA (transfer DNA) insertion line for PAPP2C (Salk_003944) had been selected in a previous study [13]. The PAPP2C knockout homozygous line was designated as papp2c and was crossed with phytochrome mutants (A-211 and B-9). The resulting F1 mutants were then selfed, and the resulting homozygous double mutants were selected from the F2 population by phenotype analysis under continuous red and far-red light conditions (17 and 8 μmol of photons m⁻² s⁻¹ respectively) and by genotyping. For generation of the PAPP2C-overexpressing line (papp2c/PAPP2C), the full-length cDNA of PAPP2C (At1g22280) was amplified by PCR, subcloned into pGEM-T easy (Promega), and eventually subcloned into the binary vector pPZP2Ha3(+) [31]. This construct was then introduced into the Agrobacterium strain GV3101, which was used to transform Arabidopsis papp2c transgenic plants via the floral-dip method [32]. Transformed lines were selected on Murashige and Skoog medium containing hygromycin (25 μg/ml). Overexpressed homozygous lines were isolated by PCR and immunoblotting with a PAPP2C-specific polyclonal antibody. The T3 progeny resulting from homozygous self-crosses were used for phenotypic characterization.

In vitro binding assay

To assay the interaction of PAPP2C with phytochrome, a pull-down experiment was performed using either full-length or truncated phytochromes and recombinant PAPP2C protein. The preparation of phytochromes was performed as described previously [27]. Phytochrome and GST (glutathione transferase)–PAPP2C (both at 1 μg) were incubated for 90 min at 4°C in binding buffer [1× PBS and 0.5% (v/v) Nonidet P40]. Glutathione resin was then added and incubated for an additional 45 min. After a quick centrifugation (8000 g for 1 min at 4°C), the precipitate was washed six times with binding buffer. The same volume of 2 × SDS sample buffer was added to the precipitate. Both supernatant and precipitate were separated on SDS/12% PAGE and analysed by immunoblotting with phytochromespecific antibodies (P25 for phyB and Oat22 for phyA), and a GST-specific monoclonal antibody (Neomarker) to detect PAPP2C. Antibody-bound proteins were detected by incubation with an HRP (horseradish peroxidase)-conjugated secondary antibody using the ECL (enhanced chemiluminescence) system (Pierce).

In vitro phytochrome phosphorylation and dephosphorylation assays

In vitro phosphorylation and dephosphorylation assays were performed using previously reported methods with minor modifications [27]. Briefly, for the autophosphorylation of phytochromes, reactions were performed in 40 μl of KP (kinase/phosphatase) buffer [50 mM Tris/HCl (pH 7.5), 10 mM MgCl2, 2 mM DTT (dithiothreitol) and 0.1 mM EGTA] containing 1 μg of oat phyA or recombinant Arabidopsis phyB. Phytochromes in KP buffer were then subjected to red light (25 μmol of photons · m⁻² · s⁻¹) or far-red light (40 μmol of photons · m⁻² · s⁻¹; GFlE-102R/FR, Good Feeling Company) irradiation for 5 min on ice prior to the reaction. Then, 1 μCi of [γ³²P]ATP (PerkinElmer Life Sciences) was added to the reaction mixture, followed by an incubation for 5 min at 30°C. For the dephosphorylation assay, phosphorylated phytochromes were obtained by incubation with 10 μl of PKA (1 unit/μl; Sigma) for 10 min at 30°C, followed by quenching with 25 mM EDTA in 20 mM Tris buffer (pH 7.5). Recombinant proteins (2 μg of GST or GST–PAPP2C) were then added to the mixture above and the reaction proceeded for 10 min at 30°C. Each reaction was eventually terminated by the addition of SDS sample buffer. Following SDS/PAGE, phosphorylated proteins were detected using a Typhoon scanner 9210 (GE Healthcare) and quantified using ImageQuant software version 5.2 (Molecular Dynamics).

In vitro PIF3 phosphorylation and dephosphorylation assays

Pfr forms of phyA and phyB samples were prepared by red-light irradiation for 5 min on ice. Then, 1 μl of 1 μM unlabelled ATP was added to the reaction mixture, which was incubated for 30 min at 30°C. Histone H1 was included in the phosphorylation reaction (1 μg of histone H1 per 1 μg of phytochrome) to stimulate phytochrome autophosphorylation activity. Recombinant GST–PAPP2C (2 μg) was then added to the autophosphorylated phytochrome-containing reaction mixture for an additional 10 min. This PAPP2C-mediated dephosphorylation reaction was terminated by the addition of 1/10 vol. of NaF/EDTA (500 mM NaF and 100 mM EDTA). Following a 5 min incubation of the reaction mixture with the addition of recombinant MBP (maltose-binding protein)–PIF3 (2 μg) and 1 μCi of [γ³²P]ATP, SDS
sample buffer was added to terminate the PIF3 phosphorylation. Phosphorylated proteins were detected as described above.

**Phenotype assay**

To investigate the effects of PAPP2C mutations upon various light signals, wild-type and mutant seeds were plated on 0.5 × Murashige and Skoog plates and grown under ambient light conditions (dark, white, red, and far-red) at 22°C for 4 days. Continuous white light (100 μmol of photons · m⁻² · s⁻¹) was provided using fluorescent FLR40D/A tubes (Osrám). Red and far-red light (17 and 8 μmol of photons · m⁻² · s⁻¹ respectively) were provided as described above. The hypocotyl lengths were photographed with a digital camera (Nikon) and analysed using NIH (National Institute of Health) Image software. To analyse the phenotypes, three independent experiments were performed under various light conditions with approx. 50 seedlings of each mutant line for each experiment. The statistical analysis was performed to determine whether the long-hypocotyl phenotype of papp2c is significant.

**GUS (β-glucuronidase) activity assay**

Histochemical assays for GUS activity in the transgenic lines were performed using intact whole seedlings as described previously [33]. Briefly, the plant tissues were incubated in 0.2 M sodium phosphate (pH 7.0) buffer containing 2% DMSO, 0.1% 5-bromo-4-chloro-3-indolyl β-d-glucuronide, 10 mM EDTA and 20% methanol at 37°C in the dark for 2–24 h as required. Incubation times were chosen to optimize the detection of the spatial distribution pattern of the GUS activity within the seedlings. After incubation, the samples were transferred to 70% ethanol to remove the chlorophylls. GUS expression patterns were observed under a SZX12 microscope (Olympus) and photographed with ImagePro Express (Olympus).

**Subcellular localization by BiFC (bimolecular fluorescence)**

*In vivo* protein–protein interactions were analysed using the BiFC method [34] via the transient transformation of onion epidermal cells. Particle bombardment was performed as described previously [35] with a PDS/He biolistic particle delivery system (Bio-Rad) with gold particles of 0.6 μm in diameter (Bio-Rad). These particles were coated with 1 μg of plasmid DNA and delivered at 9.8 cmHg vacuum with a pressure of 1100 lb/in² (1 lb/in² = 6.9 kPa) for rupture disks. The distance between the macrocarrier and the target cells was 6 cm. All manipulations including particle bombardment were performed under dim green safe light. To generate the BiFC constructs, full-length phyB and PAPP2C cDNAs were prepared by PCR and fused either to the N-terminal 155 amino acids (YFP N ) or C-terminal 86 amino acids (YFPC) of the YFP (yellow fluorescent protein)-coding region [34]. Transfected onion cells were incubated in darkness for 16 h. Protein expression was monitored at various time intervals after transfection. Transfected onion cells were subsequently mounted for microscopic analysis in the presence of DAPI (4,6-diamidino-2-phenylindole) to visualize the nucleus. The interaction and co-localization between phytochrome and PAPP2C were determined by a cooled charge-coupled device camera using an Axioplan fluorescence confocal microscope LSM 510 (Zeiss). The image analysis was carried out with LSC Image Examination (Zeiss) and Adobe Photoshop 8.0. The quantification of fluorescence signal intensity was carried out using the CellProfiler cell image analysis software version 1.0 (Broad Institute), and graphs were produced with Excel.

**RESULTS**

**PAPP2C physically interacts with both phyA and phyB**

In our previous study using immunocoupled proteomics analysis, we reported that PAPP2C, a PP type 2C, is a putative phytochrome-interacting protein [13]. To confirm the molecular association of PAPP2C with phytochrome in our present study, we performed an *in vitro* pulldown assay using a GST–PAPP2C fusion protein with either purified native oat phyA or recombinant Arabidopsis phyB (Figure 1A). In the present study, GST–PAPP2C bound strongly to both phyA and phyB and interestingly, this affinity was higher for the Pfr form of each phytochrome.

**PAPP2C specifically interacts with the PHY domain of the phytochromes**

Most of the previously reported phytochrome-interacting proteins are C-terminal interacting partners, since they were screened using this region of the phytochromes as the bait [12]. We used various deletion mutants of oat phyA to define the specific region responsible for the interaction with PAPP2C in the present study. Interestingly, the N-terminal-deleted phyA failed to interact with PAPP2C (Figure 1B) and the PHY domain within the N-terminus of oat phyA was observed to be required for the PAPP2C–phytochrome association (Figure 1C). These results indicate that PAPP2C is a binding partner for phytochrome and that the N-terminal PHY domain serves as the docking site for this association.

**PAPP2C is a novel phytochrome-associated PP**

Phosphorylation/dephosphorylation events provide mechanisms to alter the function of a variety of protein signalling molecules. PAPP2C is a member of the serine/threonine PP2C family and comprises 281 amino acids with a highly conserved catalytic domain [13,36]. The phosphatase activity of the GST–PAPP2C fusion protein was confirmed by measuring the P release from an artificial substrate, PNPP (p-nitrophenyl phosphate) (results not shown). To analyse further the relationship between the interaction with phytochrome and the activity of PAPP2C, the phosphatase activity of this novel partner toward phytochrome was determined using γ-32P-labelled oat phyA. As expected, the phosphorylated oat phyA was effectively dephosphorylated in the presence of PAPP2C (Figure 2A). Of note, the extent of Pfr dephosphorylation was 2-fold greater than that of the Pr form. We speculate that this difference comes from the higher specificity of PAPP2C for the Pfr form of phyA.

PAPP2C was found to dephosphorylate both native oat phyA and a point mutant of Ser598 (S598A), whereas an N-terminal deletion of 65 amino acids (Δ65) prevented dephosphorylation (Figure 2B). This result indicates that PAPP2C is involved in the dephosphorylation of N-terminal serine residues of phyA. Similar to phyA, phyB is autophosphorylated and the autophosphorylation site(s) reside in the NTE (N-terminal extension) region, because the NTE-deleted phyB (BA100) showed no apparent autophosphorylation activity (Supplementary Figure S1A at http://www.BiochemJ.org/bj/415/bj4150247add.htm). In addition, phyB could be dephosphorylated by PAPP2C (Supplementary Figure S1B), similar to phyA. These results showed that PAPP2C interacts and dephosphorylates both phyA and phyB *in vitro*, and the autophosphorylation site(s) of phyA and phyB are located in the NTE regions. Taken together, our results suggest the functional roles of PAPP2C in phytochrome-mediated light signal transduction through its capability to use phytochrome as a substrate [26,37].

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Figure 1 Interaction of PAPP2C with phyA and phyB

(A) In vitro pulldown assays. Native oat phyA prepared in the dark was treated with either red or far-red light. The full-length recombinant Arabidopsis phyB was reconstituted in vitro with phycocyanobilin before use. PhyA and phyB were then incubated with GST–PAPP2C or GST alone. The resulting reaction complexes were pulled down with glutathione–Sepharose 4B beads. The binding of phytochrome to GST–PAPP2C could then be detected immunologically using anti-phyA, anti-phyB and anti-GST antibodies. The histograms represent relative intensities of the bands for binding of phyA or phyB to GST–PAPP2C. (B) In vitro pulldown assays with PAPP2C and either an N-terminal or C-terminal fragment of phyA. Full-length oat phyA (FL) were prepared in the dark and treated with red light before use. The N-terminal (AN) and C-terminal (AC) regions of oat phyA was reconstituted in vitro with phycocyanobilin. The in vitro pulldown assays were performed as described in (A). (C) In vitro pulldown assays with PAPP2C and truncated phyA constructs. The full-length oat phyA (FL) was prepared as described in (B). The HKRD deletion (A875), PAS/HKRD deletion (A610), PHY/PAS/HKRD deletion (A407) and NTE-deletion mutants of recombinant oat phyA were reconstituted in vitro with phycocyanobilin. The in vitro pulldown assays were then performed as described in (A). In (B) and (C) the blots shown are of a representative experiment that was repeated three times with identical results. aa, amino acid; WB, Western blot.

PAPP2C is involved in red light signalling

We examined the functions of PAPP2C in phytochrome-mediated light-signal transduction. Northern blot analysis revealed that the PAPP2C expression levels were increased by red-light irradiation for 2 h (Figure 3A) and that the expression of light-regulated genes such as CAB1 (chlorophyll a/b binding protein 1) and CHS (chalcone synthase) is down-regulated in the PAPP2C loss-of-function mutant (papp2c) (Figure 3B). Moreover, expression analysis using the native PAPP2C promoter fused to the GUS gene further indicated that the PAPP2C expression is up-regulated by red light in planta (Figure 3C). These results confirm that this novel phosphatase is indeed involved in red-light signalling in Arabidopsis.
PAPP2C regulates the phosphorylation of phytochrome and PIF3

Figure 2 Dephosphorylation of phytochrome by PAPP2C

(A) Phytochrome was treated with red or red/far-red light prior to initiating the reactions. 32P-labelled oat phyA was then incubated with GST–PAPP2C or GST. (B) Red or red/far-red light-treated native phyA, S598A and Δ65 were labelled with γ-32P]ATP by autophosphorylation or PKA. The labelled phytochromes were then incubated with GST–PAPP2C or GST. The phosphorylated bands were visualized by autoradiography (Autorad). The results shown are representative of at least three independent experiments.

To evaluate further the physiological functions of PAPP2C in phytochrome-mediated light-signal transduction, the photoresponses of Arabidopsis were analysed using a papp2c, PAPP2C-overexpressing line (papp2c/PAPP2C), phytochrome null mutants (A-211 and B-9) and double knockout lines (papp2c/A-211 and papp2c/B-9) under various light conditions. We isolated a homozygous T-DNA knockout line for the PAPP2C gene as previously reported [13]. We further confirmed a PAPP2C overexpression line (Figure 4A) for the phenotype complementation analysis. A long-hypocotyl phenotype was shown in the papp2c mutant under continuous red light and the phenotype was complemented by PAPP2C gene overexpression, whereas this phenotype was not comparable with A-211 under continuous far-red light (Figures 4B and 4C).

Figure 3 Analysis of light-regulated PAPP2C gene expression

(A) and (B) RNA gel blot analysis of PAPP2C, CAB1 and CHS in Arabidopsis. Samples were grown for 4 days and 15 μg of total RNA was resolved for blotting and hybridization. D, samples grown in the dark; R, samples transferred from dark to red light for 2 h. (C) Histochemical assay of GUS expression in transgenic Arabidopsis plants containing the 1.5 kb PAPP2C promoter linked to GUS. Samples were grown for 4 days. The results shown were obtained from at least three independent experiments.

PAPP2C co-localizes in the nucleus with the phytochromes

The light-driven translocation of phytochromes into the nucleus is an essential component of the signal transduction pathways mediated by these photoreceptors [38]. This process also provides activated photoreceptors in the nucleus that are available for interactions with other regulatory proteins. To determine the cellular localization of the PAPP2C–phytochrome interaction, we employed a BiFC technique in onion epidermal cells [34]. The N-terminus of YFP was tagged with PAPP2C (PAPP2C–YFPN), and its C-terminus was tagged with phyB–YFPC. As reported previously, phyB was found to be mainly localized in the cytoplasm in the dark, and to be translocated into the nucleus under light illumination to form nuclear bodies [39]. The subcellular localization of the PAPP2C–YFPN and phyB–YFPC fusion products was clearly detectable in the nucleus upon red-light irradiation, whereas the fluorescent signal was not observed under darkness (Figures 5A and 5B). The interaction and co-localization between phytochrome and PAPP2C were also confirmed in BiFC analysis by using Arabidopsis protoplast cells. PhyB was actually co-localized with PAPP2C in the nucleus by red-light treatment (Supplementary Figure S2 at http://www.BiochemJ.org/bj/415/bj4150247add.htm). This result suggests that the red-light-dependent association of PAPP2C and phyB occurs in the nucleus. This result is also consistent with the findings that the PAPP2C interacts more strongly with the Pfr form of phytochrome and shows the stronger phosphatase activity towards the Pfr form of phospho-phytochrome.

PAPP2C indirectly regulates PIF3 phosphorylation via phytochrome dephosphorylation

It has been described previously that PIF3 interacts with photostabilized phytochromes and the interaction increases its phosphorylation [19]. Since PAPP2C successfully dephosphorylated phytochrome, as shown in Figure 2, we speculated whether the activity of PAPP2C towards phosphorylated phytochrome would affect the phytochrome–PIF3 relationship. We thus examined the effects of PAPP2C-controlled phytochrome upon PIF3 phosphorylation. The Pfr forms of phosphorylated phytochromes were dephosphorylated with the incubation of GST–PAPP2C. We confirmed that the PAPP2C had no direct effect on the
PIF3 dephosphorylation (results not shown). Additionally, the possible interference on the phytochrome–PIF3 interaction by PAPP2C activity was eliminated by the addition of NaF/EDTA, because metal ions are well-known requirements for the catalytic activity of type 2C PPs [40]. The phytochromes in which the phosphorylation states were decreased by GST–PAPP2C induced a significantly reduced level of the PIF3 phosphorylation (approx. 93% reduction), whereas the intact phospo-phytochrome with the GST control displayed heavy phosphorylation of MBP–PIF3 (Figure 6). Collectively, we conclude that PAPP2C indirectly regulates the PIF3 phosphorylation through controlling the phosphorylation status of the phytochromes.

**DISCUSSION**

The phytochromes are plant photoreceptors that are composed of a photosensory N-terminal and a regulatory C-terminal domain. The photosensory domain is comprised of four sub-domains; P1, PAS, GAF (cGMP-specific phosphodiesterases cyanobacterial adenylate cyclases, and formate hydrogen lyase transcription activator FhlA) and PHY [41]. The P1 domain, termed NTE, is known to be involved in the inhibition of dark reversion [42]. The BLD (bilin lyase domain) domain is composed of PAS and GAF. The PAS domain harbours the phytochrome photosensory core and is highly conserved among the phytochrome family members, except for the cyanobacterial phytochromes (the Cph2 family). The GAF domain is the bilin chromophore-binding domain. The PHY domain is also a highly conserved domain functionally linked to the GAF domain. The C-terminal domain is divided into the PRD (PAS-related domain) domain consisting of a pair of PAS repeats (PAS1 and PAS2) and the HKRD. PRD is important for nuclear localization [43]. HKRD is involved in the interaction with many phytochrome-interacting proteins and has a regulatory function in phyB signalling [12,16,44].

The in vitro assays in the present study have shown that PAPP2C interacts specifically with the PHY domain of recombinant oat phyA (Figure 1C). Although the functions of the PHY domain are not yet clear, a previous study has revealed that mutations in this region affect the proper folding of the phytochrome and disrupt the direct intermolecular interactions with downstream signalling components [45]. In addition, the PHY domain seems to be important for tuning the spectroscopic properties of the bound bilin chromophore and to play a role in stabilization of the Pfr chromophore [46]. This suggests the importance of the phytochrome-interacting partners that associate with this domain for the phytochrome signalling pathways, of which PAPP2C is one.
PAPP2C regulates the phosphorylation of phytochrome and PIF3

Figure 5 Subcellular in vivo localization of PAPP2C and phyB

(A) Nuclear co-localization of PAPP2C and phyB. Onion cells were co-transfected with PAPP2C–YFPN and phyB–YFPC and incubated in the dark for 16 h. Protein–protein interactions were confirmed by fluorescence detection of PAPP2C–YFPN and phyB–YFPC (yellow signal). Panels 1 and 4: DAPI counterstaining images; panels 2 and 5: bright field images; panels 3 and 6: YFP images. Arrows indicate nuclei (Nu). D, samples were incubated in the dark for 16 h; R, samples were transferred from dark to red light for 2 h. Similar results were obtained in three independent experiments. (B) Quantification of YFP fluorescence intensities in onion epidermal cells. Fluorescence intensity (arbitrary units) was determined using the CellProfiler software. Values are means ± S.E.M. from three independent experiments.

PAPP2C has a conserved PP2C catalytic domain [13]. PP2C is one of the serine/threonine PPs classified into the PPP family and PPM family [47]. The PPP family includes PP1, PP2A and PP2B. The PPM family includes PP2C and pyruvate dehydrogenase phosphatase [47]. Among them, PP2C is a monomeric enzyme with a highly conserved C-terminus, it requires a bivalent cation such as Mg2+ and is insensitive to okadaic acid [40,48]. In Arabidopsis, 76 putative PP2C genes have been identified [49]. Until recently, it was reported that they modulate some protein kinases and abscisic acid signalling, and also function as signal regulators that are involved in various developmental processes [36,47]. The function of PAPP2C in phytochrome-mediated light signalling was evaluated in our present study to elucidate further the physical and physiological relationship between this phosphatase and the phytochromes. PAPP2C was found to interact with both phyA and phyB in vitro and in vivo, but there was no obvious phenotypic effect upon phyA signalling in planta. We did detect long hypocotyls in the PAPP2C-knockout plants under continuous red-light conditions, which is characteristic of a phyB-defective phenotype. We also assayed other growth and developmental phenotypes such as cotyledon expansion, seed germination, flowering time, shade avoidance, and some hormonal (ABA and auxin) and abiotic stress (salt, temperature and osmosis) responses, but we could not detect any changes as a result of the loss of PAPP2C function (results not shown). These results suggest that there might be other redundant phosphatases available in the cell or unknown functions that we did not test.

The BiFC fluorescence images obtained from onion epidermal cells (Figure 5) and Arabidopsis protoplast cells (Supplementary Figure S2) clearly showed the nuclear co-localization of phytochromes and PAPP2C. Furthermore, the nuclear-localized PAPP2C seems to interact with the Pfr form of phytochrome in the nucleus upon red-light treatment.

It has been reported previously that oat phyA is phosphorylated on Ser17 and Ser598 by PKA in vitro and on Ser7 and Ser598 in vivo [21–23]. Phosphorylation of the N-terminal extension of phyA is mediated by a conformation-dependent intrinsic autophosphorylation activity, whereas the Ser598 residue in the hinge region is not autophosphorylated. The phosphorylation of Ser598 occurs via unknown phytochrome-associated kinases in a Pfr-preferential manner in vivo [23,27]. Moreover, the previously characterized phosphatase, PAPP5, dephosphorylates all three of these serine residues on oat phyA. The phosphorylation of Ser598 is also involved in the interaction of downstream signal transducers such as NDPK2 (nucleoside diphosphate kinase 2) and PIF3 [27,28].

Figure 6 PIF3 phosphorylation is regulated by the phosphatase activity of PAPP2C towards the phytochromes

The Pfr forms of phytochromes were prepared by red-light irradiation. After the incubation of GST–PAPP2C or GST with phytochromes to obtain different levels of phytochrome phosphorylation, the reaction mixture was supplemented with NaF/EDTA to inhibit the PAPP2C activity exclusively by metal-ion chelating. Following the addition of MBP–PIF3 and γ-32P[ATP, the extent of PIF3 phosphorylation was visualized by autoradiography. The loading amounts were determined by Coomassie Blue staining. The results shown are representative of at least three independent experiments.
Our results further showed that the phosphorylated Ser598 was replaced by an alanine residue (S598A), and a deletion present study by using a phyA phosphorylation mutant in which act upon Ser598. Interestingly, PAPP2C physically interacts with chrome-specific PP as it is N-terminal serine specific and does not (Figure 2B). However, PAPP2C is a somewhat different phytochrome in the nucleus. Moreover, PAPP2C indirectly controls PIF3 phosphorylation whereas the FyPP and PAPP5 interact with the C-terminal region. Furthermore, PAPP2C failed to dephosphorylate the N-terminal-serine residues of phyA (Figures 5 and 6). Hence, PIF3 is a positive regulator of hypocotyl elongation under red light conditions [50], whereas PAPP2C seems to function as a negative regulator ([50], Figures 3E and 3F).

We examined the sites of dephosphorylation by PAPP2C in our present study by using a phyA phosphorylation mutant in which Ser598 was replaced by an alanine residue (S598A), and a deletion mutant of an additional N-terminal serine-rich region (Δ65) (Figure 2B). PAPP2C failed to dephosphorylate the Δ65 mutant that had been phosphorylated by PKA. As we indicated above, PKA mainly phosphorylates the Ser598 residue of this Δ65 product. Our results further showed that the phosphorylated Ser598 of oat phyA is not dephosphorylated by PAPP2C. Hence, PAPP2C must be involved in the dephosphorylation of phytochrome N-terminal serine residues, but the specific sites remain to be examined. Similar to phyA, we found that phyB is also auto-phosphorylated in the N-terminal 100 amino acid region (i.e. NTE) and dephosphorylated by PAPP2C (Supplementary Figure S1). Our results suggest that the autophosphorylation of both phyA and phyB and their dephosphorylation by PAPP2C might be a conserved biochemical mechanism for their regulation. The N-terminal serine-rich region of oat phyA is important for the regulation of its biological activity, response to light and con-

Figure 7 Proposed functional roles of PAPP2C during the signal transfer from phytochromes to PIF3

Nuclear-localized Pfr forms of the phytochromes control the level of PIF3 phosphorylation. The phytochromes also interact with PIF3 directly in the nucleus and the augmented phosphorylation of PIF3 eventually results in the negative regulation of light signalling. Alternatively, phytochromes that interacted with PAPP2C in the nucleus prior to their binding to PIF3 are dephosphorylated and could not phosphorylate PIF3, which results in the positive regulation of light signalling.

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SUPPLEMENTARY ONLINE DATA
A novel protein phosphatase indirectly regulates phytochrome-interacting factor 3 via phytochrome

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DEPHOSPHORYLATION ASSAY OF PHYB (PHYTOCHROME B)
Recombinant full-length (phyB, 1–1172 amino acids) and NTE-deleted (BΔ100, 101–1172 amino acids) Arabidopsis phyB were prepared as previously described [1]. The autophosphorylation reaction was started by adding 0.1 mM ATP containing 10 μCi of [γ-32P]ATP (Pharmacia) and the reaction mixture was incubated at 30°C for 30 min. The reaction was terminated by the addition of 5 × SDS sample buffer. Proteins were resolved on SDS/10% PAGE and vacuum-dried for autoradiography. To verify the proteins loaded, Coomassie Blue protein staining and zinc fluorescence were performed before drying. For the dephosphorylation assay, 1.5 μg of PAPP2C was added and incubated at 30°C for 1 h after each autophosphorylation reaction.

TRANSIENT CO-EXPRESSION AND IMAGE ANALYSIS IN ARABIDOPSIS PROTOPLAST CELLS
To confirm the subcellular co-localization and interaction between phytochrome and PAPP2C in the plant cell, we attempted a BiFC (bimolecular fluorescence) analysis using Arabidopsis protoplast cells. The cDNA of phyB or PAPP2C was subcloned into BiFC vector containing either the N-terminal region of YFP (yellow fluorescent protein) for YFP-NE::PhyB or the C-terminal region of YFP for YFP-CE::PAPP2C [2]. Protoplast preparation and transient transformation were performed according to the protocols of Yoo et al. [3]. Protoplasts were prepared by incubating several leaves of 3-week-old plants in enzyme solution containing 1.5% cellulose R10, 0.4% macerozyme R10, 0.4 M mannitol, 20 mM KCl, 10 mM CaCl2 and 20 mM Mes for 3 h and the transient transformation was completed by DNA/PEG [poly(ethylene glycol)]/calcium transfection. Protoplasts were assayed for fluorescence 12–18 h after co-transfection of YFP-tagged phytochrome and PAPP2C. For light activation of phytochromes, the transfected protoplasts were treated with red light for 30 min after incubation in darkness for 18 h. Confocal imaging was carried out with a LSC-TM laser scanning microscope. Image analysis was carried out with LSC image examination and Adobe Photoshop 8.0. The quantification of fluorescence signal intensity was carried out using the CellProfiler cell image analysis software version 1.0 (Broad Institute) and the graph was produced with Microsoft Excel.

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Figure S1 Dephosphorylation of phyB by PAPP2C
(A) Autophosphorylation of full-length (PhyB) and NTE (N-terminal extension)-deleted (BΔ100) phyB. Recombinant full-length (phyB, 1–1172 amino acids) and NTE-deleted (BΔ100, 101–1172 amino acids) Arabidopsis phyB were prepared as previously described [1]. There was no autophosphorylation observed with BΔ100, indicating that the site(s) for autophosphorylation of phyB is also located within the NTE region. (B) The Pfr form of phyB was efficiently dephosphorylated by PAPP2C. For the dephosphorylation assay, PAPP2C was added and incubated at 30°C for 1 h after each autophosphorylation reaction. The molecular mass in kDa is indicated on the left-hand side. Autorad, autoradiography.
Figure S2 BiFC visualization of the interaction between phytochrome and PAPP2C in transiently transfected Arabidopsis protoplast cells

(A) Phytochrome and PAPP2C are co-localized to the nucleus with light treatment. Phytochrome (YFP-NE::PhyB) was co-expressed with PAPP2C (YFP-CE::PAPP2C) in Arabidopsis protoplast cells. The transfected protoplasts were treated with red light for 30 min after incubation under darkness for 18 h and the fluorescence image was determined by laser-scanning confocal microscopy. (B) Quantification of fluorescence intensities in transiently transfected protoplast cells. Fluorescence intensity (arbitrary units) was determined using the CellProfiler software. Values are means ± S.E.M. from three independent measurements.

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